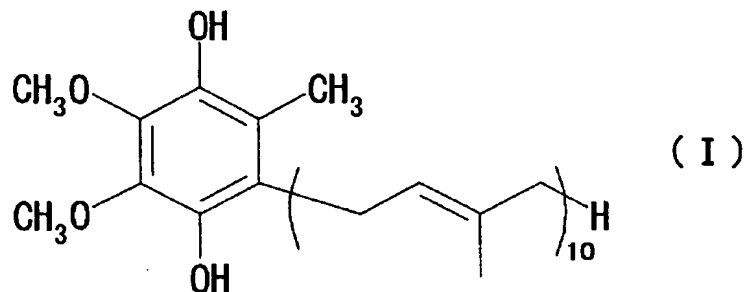


DESCRIPTION

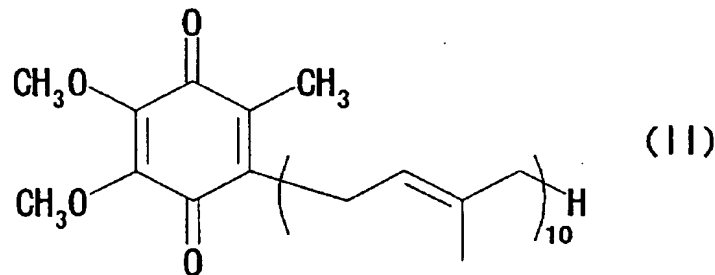
PROCESSES FOR PRODUCING COENZYME Q₁₀

TECHNICAL FIELD

The present invention relates to
a process for producing the reduced coenzyme Q₁₀
represented by the following formula (I):



and a process for producing the oxidized coenzyme Q₁₀
represented by the following formula (II):



More particularly, the present invention relates to
a process for producing reduced coenzyme Q₁₀
which comprises culturing reduced coenzyme Q₁₀-
producing microorganisms to obtain microbial cells
containing reduced coenzyme Q₁₀ at a ratio of not less than
70 mole % among the entire coenzymes Q₁₀,
optionally disrupting the microbial cells and
recovering thus-produced reduced coenzyme Q₁₀.
The present invention also relates to a process for
producing oxidized coenzyme Q₁₀ which comprises either
recovering oxidized coenzyme Q₁₀ after oxidizing the above-

mentioned microbial cells or disrupted product thereof, or recovering reduced coenzyme Q₁₀ from the above-mentioned microbial cells or disrupted product thereof to oxidize thus-obtained reduced coenzyme Q₁₀ thereafter.

5

BACKGROUND ART

The reduced coenzyme Q₁₀ (I) and the oxidized coenzyme Q₁₀ (II) are mitochondrial electron transport system-constituting factors in cells of a living body of human and deal with ATP production by working as electron carriers in oxidative phosphorylation reactions.

Conventionally, oxidized coenzyme Q₁₀ has been widely used for supplementary nutrient foods and cosmetic products in addition to pharmaceutical products as a pharmaceutically and physiologically effective substance for a variety of diseases.

On the other hand, reduced coenzyme Q₁₀ has not so much drawn attention so far; however, in these years, there has been reported that reduced coenzyme Q₁₀ is more effective in various applications than oxidized coenzyme Q₁₀.

For example, Japanese Kokai Publication Hei-10-330251 discloses an antihypercholesterolemia agent having excellent cholesterol reducing function, an antihyperlipemia agent, and an agent for curing and preventing arteriosclerosis which contain reduced coenzyme Q₁₀ as an active ingredient. In addition, Japanese Kokai Publication Hei-10-109933 discloses a pharmaceutical composition excellent in oral absorbability comprising coenzyme Q₁₀ including reduced coenzyme Q₁₀ as an active ingredient.

Furthermore, reduced coenzyme Q₁₀ is effective as an antioxidant and a radical scavenger. R. Stocker, et al. have reported that reduced coenzyme Q₁₀ prevented peroxidation of human LDL more efficiently than α -

tocopherol, lycopene and β -carotene (Proceedings of the National Academy of Science of the United States of America, vol. 88, pp. 1646-1650, 1991).

5 It has been known that oxidized coenzyme Q_{10} and reduced coenzyme Q_{10} are in a certain type of equilibrium in a living body and that oxidized coenzyme Q_{10} / reduced coenzyme Q_{10} absorbed in the living body are mutually reduced/oxidized.

10 Reduced coenzyme Q_{10} is supposedly produced by a chemical synthesis method, similarly to the process for producing oxidized coenzyme Q_{10} . But the synthesis process is supposed to be complicated, risky and costly. Moreover, in the case of chemical synthesis methods, it will be necessary to minimize the subgeneration and contamination
15 of a (Z)-isomer, which is suspiciously unsafe (Biomedical and Clinical Aspects of Coenzyme Q, vol. 3, pp. 19-30, 1981). Europe Pharmacopoeia regulates that a content of (Z)-isomer in oxidized coenzyme Q_{10} must be not more than 0.1%.

20 As another process for producing reduced coenzyme Q_{10} , it can be supposed a method of utilizing microbial cells, that is, a method for separating and recovering reduced coenzyme Q_{10} from reduced coenzyme Q_{10} -producing microorganisms. However, the reduced coenzyme Q_{10} produced
25 by the microbial cells of the above-mentioned microorganisms contains a large amount of oxidized coenzyme Q_{10} , and the separation and recovery of reduced coenzyme Q_{10} by a conventional method results in high cost.

The following are documents describing the presence
30 of reduced coenzyme Q_{10} in microbial cells and there have been known the following examples of bacteria.

(1) An example describing that at lowest 5 to 10% by weight and at highest 30 to 60% by weight of reduced coenzyme Q_{10} are present among the entire coenzymes Q_{10} in culture cells
35 of photosynthesis bacteria (Japanese Kokai Publication Sho-

57-70834).

(2) An example describing that the genus Pseudomonas is subjected to thermal extraction by an organic solvent in the presence of sodium hydroxide and pyrogallol, and the resultant is treated with 5% sodium hydrosulfite solution, and further dehydrated and concentrated to collect an acetone-soluble portion, and an oil containing reduced coenzyme Q₁₀ is obtained (Japanese Kokai Publication Sho-60-75294).

Both of the above (1) and (2) aim to convert a mixture of the obtained reduced coenzyme Q₁₀ and oxidized coenzyme Q₁₀ or the obtained reduced coenzyme Q₁₀ into oxidized coenzyme Q₁₀ by further oxidation. Thus, reduced coenzyme Q₁₀ is only described as an intermediate substance in producing oxidized coenzyme Q₁₀.

In the above (1), photosynthesis bacteria are used, the culture of which is complicated. Furthermore, in the microbial cells of the above-mentioned microorganisms, when the production of reduced coenzyme Q₁₀ is aimed at, it cannot be said that the ratio of reduced coenzyme Q₁₀ among the entire coenzymes Q₁₀ is sufficient.

The above (2) comprises a process of converting oxidized coenzyme Q₁₀ contained in a hexane phase into reduced coenzyme Q₁₀ by sodium hydrosulfite, a reducing agent (see Example 3 in Japanese Kokai Publication Sho-60-75294). Thus, the ratio of reduced coenzyme Q₁₀ among the entire coenzymes Q₁₀ in the microbial cells is not clear.

Furthermore, in both of the above (1) and (2), the production amount of coenzymes Q in culture are not described.

As described above, microbial cells containing reduced coenzyme Q₁₀ at high ratio have not been reported yet. Still less, it has not been known a fermentation production of reduced coenzyme Q₁₀ on the industrial scale, that is, a method comprising culturing microorganisms to

obtain microbial cells containing reduced coenzyme Q_{10} at high ratio among the entire coenzymes Q_{10} , and recovering reduced coenzyme Q_{10} to obtain high-purity reduced coenzyme Q_{10} .

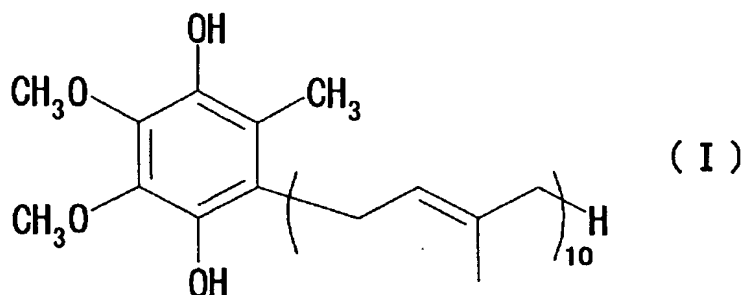
Under such circumstances, if a method for obtaining a large quantity of coenzyme Q_{10} containing reduced coenzyme Q_{10} at high ratio by culturing microorganisms is found, it can be a highly useful method for producing reduced coenzyme Q_{10} .

SUMMARY OF THE INVENTION

It is an object of the present invention to provide a process for producing reduced coenzyme Q_{10} safely and efficiently on the industrial scale by culturing reduced coenzyme Q_{10} -producing microorganisms for obtaining microbial cells containing reduced coenzyme Q_{10} at high ratio and suitably recovering reduced coenzyme Q_{10} from the microbial cells.

It is another object of the present invention to provide a process for producing oxidized coenzyme Q_{10} in simple processes by culturing reduced coenzyme Q_{10} -producing microorganisms for obtaining microbial cells containing reduced coenzyme Q_{10} at high ratio, and oxidizing the reduced coenzyme Q_{10} obtained from the microbial cells as an intermediate substance in producing oxidized coenzyme Q_{10} .

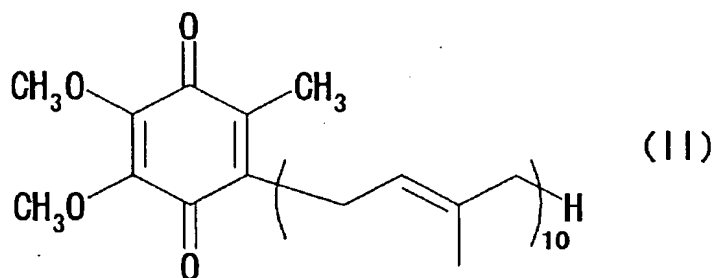
That is, the present invention relates to a process for producing the reduced coenzyme Q_{10} represented by the following formula (I):



which comprises culturing reduced coenzyme Q₁₀-
 producing microorganisms in a culture medium containing a
 10 carbon source, a nitrogen source, a phosphorus source and a
 micronutrient to obtain microbial cells containing reduced
 coenzyme Q₁₀ at a ratio of not less than 70 mole % among
 the entire coenzymes Q₁₀,

optionally disrupting the microbial cells and
 15 extracting thus-produced reduced coenzyme Q₁₀ by an
 organic solvent.

Furthermore, the present invention also relates to
 a process for producing the oxidized coenzyme Q₁₀
 represented by the following formula (II):



which comprises culturing reduced coenzyme Q₁₀-
 producing microorganisms in a culture medium containing a
 carbon source, a nitrogen source, a phosphorus source and a
 30 micronutrient to obtain microbial cells containing reduced
 coenzyme Q₁₀ at a ratio of not less than 70 mole % among
 the entire coenzymes Q₁₀,

optionally disrupting the microbial cells; and
 either oxidizing thus-produced reduced coenzyme Q₁₀
 35 to oxidized coenzyme Q₁₀ and then extracting the resultant

by an organic solvent, or extracting thus-produced reduced coenzyme Q₁₀ by an organic solvent, purifying optionally and oxidizing the resultant to oxidized coenzyme Q₁₀.

According to the processes of the present invention,
5 reduced coenzyme Q₁₀ can be produced cheaply on the industrial scale by considerably simple steps comprising culturing microorganisms and recovering reduced coenzyme Q₁₀. In addition, oxidized coenzyme Q₁₀ can also be produced by simple processes. Moreover, these coenzymes Q₁₀
10 produced by microorganisms basically do not contain (Z)-isomers thereof, and (all-E) isomers thereof can be obtained, which are same as those contained in meat, fish, etc.

15 DETAILED DISCRIPTION OF THE INVENTION

In the present invention, at first, reduced coenzyme Q₁₀-producing microorganisms are cultured to obtain microbial cells containing reduced coenzyme Q₁₀ at a ratio of not less than 70 mole %, preferably not less than 75
20 mole %, among the entire coenzymes Q₁₀ (fermentation).

The microbial cells containing reduced coenzyme Q₁₀ at such high ratio among the entire coenzymes Q₁₀ can be basically obtained by culturing microorganisms capable of producing reduced coenzyme Q₁₀ at a ratio of not less than
25 70 mole %, preferably not less than 75 mole %, among the entire coenzymes Q₁₀.

How much ratio the microorganisms can produce reduced coenzyme Q₁₀ among the entire coenzymes Q₁₀ can be evaluated, for example, by a method comprising culturing the
30 microorganisms with shaking (amplitude: 2 cm, 310 reciprocation/min) at 25°C for 72 hours in 10 mL of a culture medium [(glucose: 20 g, peptone: 5 g, yeast extract: 3g, malt extract: 3 g)/L, pH: 6.0] using a test tube (inner diameter: 21 mm, entire length: 200 mm).

35 Although the preferable culture conditions for the

fermentation production on the industrial scale will be described later, the above-mentioned culture condition is one method for standardizing the ratio of reduced coenzyme Q₁₀ produced, which microorganisms have as its ability, so
5 as to reflect the ratio within the range without having significant inaccuracies.

Under the above-mentioned culture condition, it is preferable to use microbial cells wherein a content of reduced coenzyme Q₁₀ is at a ratio of not less than 70
10 mole %, preferably not less than 75 mole %, among the entire coenzymes Q₁₀, for the present invention. It is still more preferable to use microorganisms having a productivity of reduced coenzyme Q₁₀ per unit culture medium of generally not less than 1 µg/mL, preferably not
15 less than 2 µg/mL under the above-mentioned culture condition.

The above-mentioned content of reduced coenzyme Q₁₀ and ratio of reduced coenzyme Q₁₀ among the entire coenzymes Q₁₀ can be confirmed by physically disrupting the
20 microbial cells, extracting coenzyme Q₁₀ from thus-obtained cells by an organic solvent and performing HPLC analysis. Specifically, the measurement can be carried out according to the following procedures:

- (1) The broth of microorganism is optionally concentrated,
25 10 parts by volume of the broth are displaced to a screw cap test tube (inner diameter: 16.5 mm, entire length: 130 mm), and 10 parts by volume of glass beads are added (425 to 600 µm, manufactured by SIGMA Co.);
- (2) 3 parts by volume of isopropanol and 18.5 parts by
30 volume of n-hexane relative to 10 parts by volume of the broth are added under a nitrogen atmosphere;
- (3) microbial cell disruption and extraction are carried out by vigorously shaking of the mixture for 3 minutes under a nitrogen atmosphere; and
- 35 (4) the obtained hydrophobic organic solvent phase (n-

hexane phase) is evaporated (bath temperature: 40°C) under reduced pressure to analyze the resultant by HPLC.

Column: YMC-Pack 4.6 × 250 mm (manufactured by YMC. Co., Ltd.)

5 Mobile phase: methanol/n-hexane = 85/15

Flow rate: 1 mL/min,

Detection: UV 275 nm

Retention time: reduced coenzyme Q₁₀ 13.5 min

oxidized coenzyme Q₁₀ 22.0 min

10 The above-mentioned measurement method is provided for the obtained result to reflect the reduced coenzyme Q₁₀ content and the ratio of reduced coenzyme Q₁₀ among the entire coenzymes Q₁₀ as accurate as possible, and to standardize the content and the ratio of reduced coenzyme
15 Q₁₀, which can be guaranteed at the minimum. This method has been demonstrated, by several experimentations performed by the present inventors, easy and suitable to be carried out.

As the above-mentioned reduced coenzyme Q₁₀-producing
20 microorganisms to be used in the present invention, bacteria, yeast and fungi may be used without any specific limitation. As specific examples of the above-mentioned microorganisms, there may be mentioned, for example, microorganisms of the genus Agrobacterium, the genus
25 Aspergillus, the genus Acetobacter, the genus Aminobacter, the genus Agromonas, the genus Acidiphilium, the genus Bulleromyces, the genus Bullera, the genus Brevundimonas, the genus Cryptococcus, the genus Chionosphaera, the genus Candida, the genus Cerinosterus, the genus Exisophiala, the
30 genus Exobasidium, the genus Fellomyces, the genus Filobasidiella, the genus Filobasidium, the genus Geotrichum, the genus Graphiola, the genus Gluconobacter, the genus Kockovaella, the genus Kurtzmanomyces, the genus Lalaria, the genus Leucosporidium, the genus Legionella,
35 the genus Methylobacterium, the genus Mycoplasma, the genus

Oosporidium, the genus Pseudomonas, the genus Pseudozyma,
 the genus Paracoccus, the genus Petromyc, the genus
Rhodotorula, the genus Rhodosporidium, the genus Rhizomonas,
 the genus Rhodobium, the genus Rhodoplanes, the genus
 5 Rhodopseudomonas, the genus Rhodobacter, the genus
Sporobolomyces, the genus Sporidiobolus, the genus
Saitoella, the genus Schizosaccharomyces, the genus
Sphingomonas, the genus Sporotrichum, the genus
Sympodiomyces, the genus Sterigmatosporidium, the genus
 10 Tapharina, the genus Tremella, the genus Trichosporon, the
 genus Tilletiaria, the genus Tilletia, the genus
Tolyposporium, the genus Tilletiopsis, the genus Ustilago,
 the genus Udeniomyces, the genus Xanthophyllomyces, the genus
Xanthobacter, the genus Paecilomyces, the genus Acremonium,
 15 the genus Hyphomonus, and the genus Rhizobium.

In terms of the culture easiness and productivity,
 bacteria (preferably nonphotosynthetic bacteria) and yeast
 are preferred. As the bacteria, there may be mentioned,
 for example, the genus Agrobacterium, the genus
 20 Gluconobacter and the like. As the yeast, there may be
 mentioned, for example, the genus Schizosaccharomyces, the
 genus Saitoella and the like.

As preferable species, there may be mentioned, for
 example, Agrobacterium tumefaciens IF013263, Agrobacterium
 25 radiobacter ATCC4718, Aspergillus clavatus JCM1718,
Acetobacter xylinum IF015237, Aminobacter aganouensis
 JCM7854, Agromonas oligotrophica JCM1494, Acidiphilium
multivorum JCM8867, Bulleromyces albus IF01192, Bullera
armeniaca IF010112, Brevundimonas diminuta JCM2788,
 30 Cryptococcus laurentii IF00609, Chionosphaera apobasidialis
 CBS7430, Candida curvata ATCC10567, Cerinosterus luteoalbus
 JCM2923, Exisophiala alcalophila JCM12519, Exobasidium
gracile IF07788, Fellomyces fuzhouensis IF010374,
Filobasidiella neoformans CBS132, Filobasidium
 35 capsuloigenum CBS1906, Geotrichum capitatum JCM6258,

Graphiola cylindrica IF06426, Gluconobacter suboxydans
 IF03257, Kockovaella imperatae JCM7826, Kurtzmanomyces
nectairei IF010118, Lalaria cerasi CBS275.28,
 5 Leucosporidium scottii IF01212, Legionella anisa JCM7573,
Methylobacterium extorquens JCM2802, Mycoplana ramosa
 JCM7822, Oosporidium margaritifera CBS2531, Pseudomonas
denitrificans IAM 12023, Pseudomonas shuylkilliensis IAM
 1092, Pseudozyma aphidis CBS517.23, Paracoccus denitrificans
 JCM6892, Petromyces alliaceus IF07538, Rhodotorula glutinis
 10 IF01125, Rhodotorula minuta IF00387, Rhodospiridium
diobovatum ATCC1830, Rhizomonas suberifaciens IF015212,
Rhodobium orientis JCM9337, Rhodoplanes elegans JCM9224,
Rhodopseudomonas palustris JCM2524, Rhodobacter capsulatus
 SB1003, Sporobolomyces holsaticus IF01034, Sporobolomyces
 15 pararoseus IF00471, Sporidiobolus johnsonii IF01840,
Saitoella complicata IF010748, Schizosaccharomyces pombe
 IF00347, Sphingomonas parapaucimobilis IF015100,
Sporotrichum cellulophilum ATCC20493, Sympodiomyces
paphiopedili JCM8318, Sterigmatosporidium polymorphum
 20 IF010121, Sphingomonas adhesiva JCM7370, Tapharina
caerulescens CBS351.35, Tremella mesenterica ATCC24438,
Trichosporon cutaneum IF01198, Tilletiaria anomala
 CBS436.72, Tilletia caries JCM1761, Tolyposporium bullatum
 JCM2006, Tilletiopsis washintonensis CBS544, Ustilago
 25 esculenta IF09887, Udeniomyces megalosporus JCM5269,
Xanthophyllomyces dendrorhous IF010129, Xanthobacter flavus
 JCM1204, Paecilomyces lilacinus ATCC10114, Acremonium
chrysogenum ATCC11550, Hyphomonas hirschiana ATCC33886,
Rhizobium meliloti ATCC9930, and the like.

30 As the reduced coenzyme Q₁₀-producing microorganisms,
 not only the wild species of the above-mentioned
 microorganisms but also microorganisms in which the
 transcription and translation activities of the genes
 relevant to the biosynthesis of reduced coenzyme Q₁₀ in the
 35 above-mentioned microorganisms, or the enzyme activity of

the expressed protein are modified or improved can be used preferably, for example.

As the means for modifying or improving the transcription and translation activities of the genes or the enzyme activity of the expressed protein, there may be mentioned gene recombination (including gene improvement, amplification and destruction by itself, external gene introduction, and gene improvement and proliferation of thus-introduced external genes) and mutagenesis by mutagens. In particular, the mutagenesis by mutagens is preferred.

The more preferable microorganisms usable for the present invention are microorganisms containing reduced coenzyme Q_{10} at a ratio of not less than 70 mole %, preferably not less than 75 mole %, more preferably not less than 80 mole %, still more preferably not less than 85 mole %, and particularly preferably not less than 90 mole %, among the entire coenzymes Q_{10} in the case where the above-mentioned modified or improved microorganisms, preferably microorganisms mutated by mutagens, are evaluated by the above-mentioned proliferation method and the measurement method. In the fermentation production on the industrial scale, it is preferable to use microorganisms having a productivity of reduced coenzyme Q_{10} per unit culture medium of not less than 1 $\mu\text{g/mL}$, preferably not less than 2 $\mu\text{g/mL}$, more preferably not less than 3 $\mu\text{g/mL}$, still more preferably not less than 5 $\mu\text{g/mL}$, particularly preferably not less than 10 $\mu\text{g/mL}$, much more preferably not less than 15 $\mu\text{g/mL}$, and most preferably not less than 20 $\mu\text{g/mL}$.

The mutagenesis may be carried out by a single mutagenesis; however, mutagenesis is preferably carried out not less than 2 times. That is because it was found that the productivity of reduced coenzyme Q_{10} can be improved in the respective mutagenesis steps. It is needless to say that the candidates of the microbial cells to be mutated are, generally, those having a productivity of reduced

coenzyme Q₁₀ as high as possible in the case where the evaluation is carried out by the above-mentioned proliferation method and measurement method.

5 The mutagenesis can be carried out by using optional and proper mutagens. The term "mutagen" encompasses, in a board definition, not only chemical agents having mutagenesis effects, for example, but also treatments such as UV radiation having mutagenesis effects. As examples of proper mutagens, there may be mentioned ethyl
10 methanesulfonate, UV radiation, N-methyl-N'-nitro-N-nitrosoguanidine, nucleotide base analogues such as bromouracil, and acridines; however, they are not limited to these examples.

According to a conventional mutagenesis technique,
15 successively to the mutagenesis, a proper selection of microbial cells having high productivity of reduced coenzyme Q₁₀ is carried out. For that, the culture obtained from a single colony should be evaluated, for example, by the above-mentioned proliferation method and measurement
20 method. Since a reduced coenzyme Q₁₀ crystal forms a white solid layer or a colorless liquid phase, a productivity of reduced coenzyme Q₁₀ can be suitably evaluated by the above-mentioned measurement method at the time of selection of the colony.

25 In the processes of the present invention, high productivity of reduced coenzyme Q₁₀ in the fermentation production on the industrial scale can be achieved partially by using the microbial cells containing reduced coenzyme Q₁₀ at a ratio of not less than 70 mole % among
30 the entire coenzymes Q₁₀ and, partially, by using the suitable conditions of culture (fermentation) for increasing a productivity of reduced coenzyme Q₁₀ per unit culture medium as described below. It is particularly preferable to combinedly use suitable microbial cells
35 described above and the suitable conditions of culture

(fermentation) as described below.

The culture is carried out, in general, in a culture medium containing major nutrients and micronutrients suited for microorganism proliferation. As the above-mentioned
5 nutrients, there may be mentioned, for example, carbon sources (e.g. hydrocarbons such as glucose, sucrose, maltose, starch, corn syrup and molasses; alcohols such as methanol and ethanol), nitrogen sources (e.g. corn steep liquor, ammonium sulfate, ammonium phosphate, ammonium
10 hydroxide, urea and peptone), phosphorus sources (e.g. ammonium phosphate and phosphoric acid) and micronutrients (e.g. minerals such as magnesium, potassium, zinc, copper, iron, manganese, molybdenum, sulfuric acid and hydrochloric acid; vitamins such as biotin, desthiobiotin and vitamin
15 B1; amino acids such as alanine and histidine; and natural raw materials containing vitamins such as yeast extract and malt extract); however, these are not limitative ones, and commonly used ones may be used. Incidentally, in natural components of a culture medium, such as yeast extract,
20 phosphorus sources such as phosphates are contained. The above-mentioned nutrients can be appropriately used in combination.

The culture is generally carried out at a temperature range of 15 to 45°C, preferably 20 to 37°C. If it is below
25 15°C, the proliferation speed of microorganisms tends to be too slow to allow the industrial production and at high temperatures exceeding 45°C, the viability of microorganisms tends to be easily hindered.

In general, the culture is carried out at a pH range
30 of 4 to 9, preferably 5 to 8. If the pH is not more than 3 or not less than 10, proliferation of microorganisms tends to be easily inhibited.

In the fermentation production on the industrial scale, although it depends on the microorganism species,
35 the concentration of the carbon sources (including the

produced alcohols) during the culture is preferably controlled to a concentration that no adverse effects are substantially caused on the productivity of reduced coenzyme Q₁₀. Accordingly, it is preferable to control the culture so as to have the concentration of the carbon sources that no adverse effects are substantially caused on the productivity of reduced coenzyme Q₁₀, that is, generally to not more than 20 g/L, preferably not more than 5 g/L, and more preferably not more than 2 g/L in the broth.

To control the concentration of the carbon sources, a fed batch culture method is preferably used. The carbon source concentration in the broth can be controlled by adjusting the supply of nutrient sources (especially carbon sources) based on the culture control indexes such as pH, the dissolved oxygen concentration (DO) or the remaining saccharide concentration. Although it depends on the microorganism species, the supply of the nutrient sources may be started from the initial stage of the culture or during the culture. The supply of the nutrient sources may be continuous or intermittent. Incidentally, in supplying the nutrient sources, it is preferable to supply the above-mentioned carbon sources to the culture medium separately from other components.

The culture can be completed at the point when a desired amount of reduced coenzyme Q₁₀ is produced. The culture duration is not particularly limited and it is generally 20 to 200 hours.

The above-mentioned culture is generally carried out aerobically. The term "aerobically" means a condition that oxygen is supplied so as not to cause oxygen limitation (oxygen deficiency) during the culture, and preferably a condition that oxygen is supplied sufficiently so as not to cause substantial oxygen limitation during the culture. The culture is carried out generally under an aeration condition, preferably under an aeration and stirring

condition.

By using the above-mentioned microorganisms and culture conditions, it becomes possible to obtain microbial cells containing reduced coenzyme Q_{10} at a ratio of not less than 70 mole %, preferably not less than 75 mole % among the entire coenzymes Q_{10} . Furthermore, the productivity of reduced coenzyme Q_{10} of as high as not less than 1 $\mu\text{g/mL}$, preferably not less than 2 $\mu\text{g/mL}$, and still more preferably not less than 3 $\mu\text{g/mL}$ can be obtained.

Next, recovery of the reduced coenzyme Q_{10} produced by the above-mentioned culture will be described.

In the present invention, an efficient production of reduced coenzyme Q_{10} on the industrial scale is made to be possible partially by the above-mentioned suitable culture and partially by the suitable recovery process of reduced coenzyme Q_{10} as described below.

Recovery of reduced coenzyme Q_{10} is carried out by extraction from the microbial cells obtained by the above-mentioned culture using an organic solvent.

In the extraction, cells can be disrupted optionally. The cell disruption contributes to the efficient extraction of the reduced coenzyme Q_{10} produced and accumulated in cells. It is needless to say that the cell disruption and extraction can be carried out at the same time.

Incidentally, "disruption" in the present invention may be carried out to the extent that the surface structure such as a cell wall is broken so as to make extraction of reduced coenzyme Q_{10} possible; therefore, it is not necessary that microbial cells are torn or fragmentated.

The above-mentioned cell disruption is not necessarily required in the case of bacteria. However, in the case of yeast or fungi, the cell disruption is generally required and, when cells are not disrupted, it becomes difficult to efficiently recover the reduced coenzyme Q_{10} produced and accumulated in the cells.

The above-mentioned disruption of microbial cells can be carried out by the following one or several disruption methods in optional order. As the disruption method, there may be mentioned, for example, a physical treatment, a
5 chemical treatment, an enzymic treatment as well as a heating treatment, an autolysis, an osmolysis, a plasmoptysis and the like.

The above-mentioned physical treatment can be carried out, for example, by using a high pressure homogenizer, an
10 ultrasonic homogenizer, a French press, a ball mill and the like or using them in combination.

The above-mentioned chemical treatment can be carried out, for example, by using an acid (preferably a strong acid) such as hydrochloric acid and sulfuric acid, a base
15 (preferably a strong base) such as sodium hydroxide and potassium hydroxide and the like or using them in combination.

The above-mentioned enzymic treatment can be carried out, for example, by using lysozyme, zymolyase, glucanase,
20 Novozyme, protease, cellulase and the like or by using them appropriately in combination.

The above-mentioned heating treatment can be carried out, for example, by heating to the temperature range of 60 to 100°C for about 30 minutes to 3 hours.

25 The above-mentioned autolysis can be carried out, for example, by treatment with a solvent such as ethyl acetate.

The osmolysis or the plasmoptysis for disrupting cells by treating cells with a solution having a different salt concentration from that in the cells are often
30 combinedly used with the above-mentioned physical treatment, chemical treatment, enzymic treatment, heating treatment, autolysis and/or the like since the above lytic method alone is insufficient in the disruption effect.

As the cell disruption method as a pretreatment of
35 extraction and recovery of reduced coenzyme Q₁₀, among the

above-mentioned disruption methods, the physical treatment, the chemical treatment (particularly, an acid treatment and preferably the one with a strong acid (e.g. an acid having a pKa value of not more than 2.5 in the form of an aqueous solution) under the condition that reduced coenzyme Q₁₀ is protected from an oxidation reaction as described below) and the heating treatment are preferred. From the viewpoint of disruption efficiency, the physical treatment is more preferred.

A conventional cell disruption method and coenzyme Q₁₀ extraction method, specifically, a method comprising extracting coenzyme Q₁₀ by an organic solvent in the presence of sodium hydroxide and pyrogallol has problems in terms of cost, waste treatment, safety in effective utilization of waste microorganisms (waste cells) such as recovery of protein, and the like. However, the cell disruption method, particularly the physical treatment method of the present invention, does not cause subgeneration of a large quantity of salts by neutralization, and is a suitable method from a viewpoint of the waste treatment and the effective utilization of waste microorganisms (waste cells).

The form of the microbial cells to be used for the above-mentioned cell disruption may be a broth, a concentrated broth, microbial cells collected as wet cells from the broth, a product obtained by washing them, a suspension of the wet cells in a solvent (including, for example, water, physiological saline solution, buffers and the like), dry cells obtained by drying the above-mentioned wet cells, a suspension of the dry cells in a solvent (including, for example, water, physiological saline solution, buffers and the like), and the like. Preferred is an aqueous suspension of microbial cells, and in terms of operability and the like, more preferred are the broth, the concentrated broth, and the product obtained by washing

them.

The form of the above-mentioned microbial cells or disrupted product thereof to be used for extraction and recovery of reduced coenzyme Q_{10} is, similarly as described above, not particularly limited and may be wet cells/dry cells of the microbial cells/disrupted product thereof. Preferably, it is an aqueous suspension of the microbial cells or disrupted product thereof, and more preferably the broth, the concentrated and/or washed broth, or solutions obtained by disrupting them (each of them is an aqueous suspension).

The cell concentration in the above-mentioned suspension of the microbial cells or disrupted product thereof is not particularly limited and is generally 1 to 25% by weight on the basis of dry weight. Preferably, it is 10 to 20% by weight in terms of cost.

Reduced coenzyme Q_{10} can be recovered by extracting the microbial cells and disrupted product thereof obtained in such a manner by an organic solvent.

As the organic solvent to be used for the extraction, there may be mentioned hydrocarbons, fatty acid esters, ethers, alcohols, fatty acids, ketones, nitrogen compounds (including nitriles and amides), sulfur compounds and the like.

Particularly, in extracting reduced coenzyme Q_{10} , in terms of protection from oxidation by a molecular oxygen, at least one species of hydrocarbons, fatty acid esters, ethers, and nitriles is preferably used. Among them, hydrocarbons and fatty acid esters are particularly preferable, and hydrocarbons are most preferable.

On the industrial production scale, complete oxygen elimination is very difficult to be achieved and, furthermore, fairly long periods of time are required for individual operations, unlike laboratory scale production, so that residual oxygen exerts a great adverse effect. The

oxidation in question is directly connected to a subgeneration of oxidized coenzyme Q_{10} from reduced coenzyme Q_{10} . Accordingly, use of the above-mentioned organic solvent (such as hydrocarbons, fatty acid esters, ethers, and nitriles) with high oxidation prevention effect in the extraction of reduced coenzyme Q_{10} assists an efficient extraction.

The hydrocarbons are not particularly restricted, but there may be mentioned, for example, aliphatic hydrocarbons, aromatic hydrocarbons, halogenated hydrocarbons, and the like. Preferred are aliphatic hydrocarbons and aromatic hydrocarbons, and more preferred are aliphatic hydrocarbons.

The aliphatic hydrocarbons are not particularly restricted, and may be cyclic or acyclic, or saturated or unsaturated. However, generally, saturated ones are preferably used. Usually, ones containing 3 to 20 carbon atoms, preferably 5 to 12 carbon atoms, and more preferably 5 to 8 carbon atoms are used. As specific examples, there may be mentioned, for example, propane, butane, isobutane, pentane, 2-methylbutane, hexane, 2-methylpentane, 2,2-dimethylbutane, 2,3-dimethylbutane, heptane, heptane isomers (e.g. 2-methylhexane, 3-methylhexane, 2,3-dimethylpentane, 2,4-dimethylpentane), octane, 2,2,3-trimethylpentane, isooctane, nonane, 2,2,5-trimethylhexane, decane, dodecane, 2-pentene, 1-hexene, 1-heptene, 1-octene, 1-nonene, 1-decene, cyclopentane, methylcyclopentane, cyclohexane, methylcyclohexane, ethylcyclohexane, p-menthane, cyclohexene, and the like. Preferred are pentane, 2-methylbutane, hexane, 2-methylpentane, 2,2-dimethylbutane, 2,3-dimethylbutane, heptane, heptane isomers (e.g. 2-methylhexane, 3-methylhexane, 2,3-dimethylpentane, 2,4-dimethylpentane), octane, 2,2,3-trimethylpentane, isooctane, nonane, 2,2,5-trimethylhexane, decane, dodecane, cyclopentane, methylcyclopentane, cyclohexane, methylcyclohexane, ethylcyclohexane, p-menthane, and the

like. More preferred are pentane, 2-methylbutane, hexane, 2-methylpentane, 2,2-dimethylbutane, 2,3-dimethylbutane, heptane, heptane isomers (e.g. 2-methylhexane, 3-methylhexane, 2,3-dimethylpentane, 2,4-dimethylpentane),
5 octane, 2,2,3-trimethylpentane, isooctane, cyclopentane, methylcyclopentane, cyclohexane, methylcyclohexane, ethylcyclohexane, and the like.

Generally, heptanes, not only heptane but also heptane isomers such as methylcyclohexane having 7 carbon
10 atoms and a mixture thereof are preferably used. More preferred are pentanes (e.g. pentane and the like) having 5 carbon atoms, hexanes (e.g. hexane, cyclohexane and the like) having 6 carbon atoms, and heptanes (e.g. heptane, methylcyclohexane and the like) having 7 carbon atoms.
15 Particularly preferred are heptanes (e.g. heptane, methylcyclohexane and the like) in terms of especially high protection effect from oxidation, and the most preferred is heptane.

The aromatic hydrocarbons are not particularly
20 restricted, but generally ones containing 6 to 20 carbon atoms, preferably 6 to 12 carbon atoms, and more preferably 7 to 10 carbon atoms are used. As specific examples, there may be mentioned, for example, benzene, toluene, xylene, o-xylene, m-xylene, p-xylene, ethylbenzene, cumene,
25 mesitylene, tetralin, butylbenzene, p-cymene, cyclohexylbenzene, diethylbenzene, pentylbenzene, dipentylbenzene, dodecylbenzene, styrene, and the like. Preferred are toluene, xylene, o-xylene, m-xylene, p-xylene, ethylbenzene, cumene, mesitylene, tetralin, butylbenzene,
30 p-cymene, cyclohexylbenzene, diethylbenzene, pentylbenzene and the like. More preferred are toluene, xylene, o-xylene, m-xylene, p-xylene, cumene, tetralin and the like, and most preferred is cumene.

The halogenated hydrocarbons are not particularly
35 restricted, and may be cyclic or acyclic, or saturated or

unsaturated. However, acyclic ones are preferably used in general. Usually, more preferred are chlorinated hydrocarbons and fluorinated hydrocarbons, and chlorinated hydrocarbons are still more preferred. Additionally, ones
 5 containing 1 to 6 carbon atoms, preferably 1 to 4 carbon atoms, and more preferably 1 to 2 carbon atoms are suitably used. As specific examples, for example, there may be mentioned dichloromethane, chloroform, carbon tetrachloride, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-
 10 trichloroethane, 1,1,2-trichloroethane, 1,1,1,2-tetrachloroethane, 1,1,2,2-tetrachloroethane, pentachloroethane, hexachloroethane, 1,1-dichloroethylene, 1,2-dichloroethylene, trichloroethylene, tetrachloroethylene, 1,2-dichloropropane, 1,2,3-
 15 trichloropropane, chlorobenzene, 1,1,1,2-tetrafluoroethane, and the like. Preferred are dichloromethane, chloroform, carbon tetrachloride, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1,1,2-trichloroethane, 1,1-dichloroethylene, 1,2-dichloroethylene, trichloroethylene, chlorobenzene, 1,1,1,2-tetrafluoroethane,
 20 and the like. More preferred are dichloromethane, chloroform, 1,2-dichloroethylene, trichloroethylene, chlorobenzene, 1,1,1,2-tetrafluoroethane and the like.

The fatty acid esters are not particularly restricted,
 25 but there may be mentioned, for example, propionates, acetates, formates, and the like. Preferred are acetates and formates, and more preferred are acetates. Ester functional groups thereof are not particularly restricted, but, in general, preferred are alkyl esters having 1 to 8
 30 carbon atoms and aralkyl esters having 7 to 12 carbon atoms, more preferred are alkyl esters having 1 to 6 carbon atoms, and still more preferred are alkyl esters having 1 to 4 carbon atoms.

As specific examples of the propionates, there may be
 35 mentioned, for example, methyl propionate, ethyl propionate,

butyl propionate, isopentyl propionate, and the like. Preferred are ethyl propionate and the like.

As specific examples of the acetates, there may be mentioned, for example, methyl acetate, ethyl acetate, 5 propyl acetate, isopropyl acetate, butyl acetate, isobutyl acetate, sec-butyl acetate, pentyl acetate, isopentyl acetate, sec-hexyl acetate, cyclohexyl acetate, benzyl acetate, and the like. Preferred are methyl acetate, ethyl acetate, propyl acetate, isopropyl acetate, butyl acetate, 10 isobutyl acetate, sec-butyl acetate, pentyl acetate, isopentyl acetate, sec-hexyl acetate, cyclohexyl acetate, and the like. More preferred are methyl acetate, ethyl acetate, propyl acetate, isopropyl acetate, butyl acetate, isobutyl acetate, and the like. Most preferred is ethyl 15 acetate.

As specific examples of the formates, there may be mentioned, for example, methyl formate, ethyl formate, propyl formate, isopropyl formate, butyl formate, isobutyl formate, sec-butyl formate, pentyl formate, and the like. 20 Preferred are methyl formate, ethyl formate, propyl formate, butyl formate, isobutyl formate, pentyl formate, and the like. Most preferred is ethyl formate.

The ethers are not particularly restricted, and may be cyclic or acyclic, or saturated or unsaturated. But 25 saturated ones are preferably used in general. Generally, ones containing 3 to 20 carbon atoms, preferably 4 to 12 carbon atoms and more preferably 4 to 8 carbon atoms are used. As specific examples, there may be mentioned, for example, diethyl ether, methyl tert-butyl ether, dipropyl 30 ether, diisopropyl ether, dibutyl ether, dihexyl ether, ethyl vinyl ether, butyl vinyl ether, anisol, phenetole, butyl phenyl ether, methoxytoluene, dioxane, furan, 2-methylfuran, tetrahydrofuran, tetrahydropyran, ethylene glycol dimethyl ether, ethylene glycol diethyl ether, 35 ethylene glycol dibutyl ether, ethylene glycol monomethyl

ether, ethylene glycol monoethyl ether, ethylene glycol monobutyl ether, and the like. Preferred are diethyl ether, methyl tert-butyl ether, dipropyl ether, diisopropyl ether, dibutyl ether, dihexyl ether, anisol, phenetole, butyl
 5 phenyl ether, methoxytoluene, dioxane, 2-methylfuran, tetrahydrofuran, tetrahydropyran, ethylene glycol dimethyl ether, ethylene glycol diethyl ether, ethylene glycol dibutyl ether, ethylene glycol monomethyl ether, ethylene glycol monoethyl ether, and the like. More preferred are
 10 diethyl ether, methyl tert-butyl ether, anisol, dioxane, tetrahydrofuran, ethylene glycol monomethyl ether, ethylene glycol monoethyl ether, and the like. Still more preferred are diethyl ether, methyl tert-butyl ether, anisol, and the like, and most preferred is methyl tert-butyl ether.

15 The alcohols are not particularly restricted but may be cyclic or acyclic, or saturated or unsaturated. Saturated ones are generally preferred, however. Generally, ones containing 1 to 20 carbon atoms, more preferably 1 to 12 carbon atoms, and still more preferably 1 to 6 carbon
 20 atoms are used. Among them, monohydric alcohols containing 1 to 5 carbon atoms, dihydric alcohols containing 2 to 5 carbon atoms, and trihydric alcohols containing 3 carbon atoms are preferred.

As specific examples of these alcohols, there may be
 25 mentioned, for example, monohydric alcohols such as methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutyl alcohol, tert-butyl alcohol, 1-pentanol, 2-pentanol, 3-pentanol, 2-methyl-1-butanol, isopentyl alcohol, tert-pentyl alcohol, 3-methyl-2-butanol, neopentyl
 30 alcohol, 1-hexanol, 2-methyl-1-pentanol, 4-methyl-2-pentanol, 2-ethyl-1-butanol, 1-heptanol, 2-heptanol, 3-heptanol, 1-octanol, 2-octanol, 2-ethyl-1-hexanol, 1-nonanol, 1-decanol, 1-undecanol, 1-dodecanol, allyl alcohol, propargyl alcohol, benzyl alcohol, cyclohexanol, 1-
 35 methylcyclohexanol, 2-methylcyclohexanol, 3-

methyleyclohexanol, 4-methyleyclohexanol, and the like;
 dihydric alcohols such as 1,2-ethanediol, 1,2-propandiol,
 1,3-propandiol, 1,2-butanediol, 1,3-butanediol, 1,4-
 butanediol, 2,3-butanediol, 1,5-pentanediol, and the like;
 5 and trihydric alcohols such as glycerol, and the like.

As the monohydric alcohols, preferred are methanol,
 ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol,
 isobutyl alcohol, tert-butyl alcohol, 1-pentanol, 2-
 pentanol, 3-pentanol, 2-methyl-1-butanol, isopentyl alcohol,
 10 tert-pentyl alcohol, 3-methyl-2-butanol, neopentyl alcohol,
 1-hexanol, 2-methyl-1-pentanol, 4-methyl-2-pentanol, 2-
 ethyl-1-butanol, 1-heptanol, 2-heptanol, 3-heptanol, 1-
 octanol, 2-octanol, 2-ethyl-1-hexanol, 1-nonanol, 1-decanol,
 1-undecanol, 1-dodecanol, benzyl alcohol, cyclohexanol, 1-
 15 methyleyclohexanol, 2-methyleyclohexanol, 3-
 methyleyclohexanol, 4-methyleyclohexanol, and the like.
 More preferred are methanol, ethanol, 1-propanol, 2-
 propanol, 1-butanol, 2-butanol, isobutyl alcohol, tert-
 butyl alcohol, 1-pentanol, 2-pentanol, 3-pentanol, 2-
 20 methyl-1-butanol, isopentyl alcohol, tert-pentyl alcohol,
 3-methyl-2-butanol, neopentyl alcohol, 1-hexanol, 2-methyl-
 1-pentanol, 4-methyl-2-pentanol, 2-ethyl-1-butanol,
 cyclohexanol, and the like. Still more preferred are
 methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-
 25 butanol, isobutyl alcohol, tert-butyl alcohol, 1-pentanol,
 2-pentanol, 3-pentanol, 2-methyl-1-butanol, isopentyl
 alcohol, tert-pentyl alcohol, 3-methyl-2-butanol, neopentyl
 alcohol, and the like. Particularly preferred are methanol,
 ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol,
 30 isobutyl alcohol, 2-methyl-1-butanol, isopentyl alcohol,
 and the like. Most preferred is 2-propanol.

As the dihydric alcohols, preferred are 1,2-
 ethanediol, 1,2-propandiol, 1,3-propandiol, and the like.
 Most preferred is 1,2-ethanediol. As the trihydric
 35 alcohols, glycerol is preferred.

As fatty acids, there may be mentioned, for example, formic acid, acetic acid, propionic acid, and the like. Preferred are formic acid and acetic acid, and most preferred is acetic acid.

5 The ketones are not particularly restricted, and ones having 3 to 6 carbon atoms are preferably used. As specific examples, there may be mentioned, for example, acetone, methyl ethyl ketone, methyl butyl ketone, methyl isobutyl ketone, and the like. Preferred are acetone and
10 methyl ethyl ketone, and most preferred is acetone.

The nitriles are not particularly restricted, and may be cyclic or acyclic, or saturated or unsaturated. However, saturated ones are preferably used in general. Generally, ones containing 2 to 20 carbon atoms, preferably 2 to 12
15 carbon atoms, and more preferably 2 to 8 carbon atoms are used.

As specific examples, there may be mentioned, for example, acetonitrile, propiononitrile, malononitrile, butyronitrile, isobutyronitrile, succinonitrile,
20 valeronitrile, glutaronitrile, hexanenitrile, heptylcyanide, octylcyanide, undecanenitrile, dodecanenitrile, tridecanenitrile, pentadecanenitrile, stearonitrile, chloroacetonitrile, bromoacetonitrile, chloropropiononitrile, bromopropiononitrile,
25 methoxyacetonitrile, methyl cyanoacetate, ethyl cyanoacetate, tolunitrile, benzonitrile, chlorobenzonitrile, bromobenzonitrile, cyanobenzoic acid, nitrobenzonitrile, anisonitrile, phthalonitrile, bromotolunitrile, methyl cyanobenzoate, methoxybenzonitrile, acetylbenzonitrile,
30 naphthonitrile, biphenylcarbonitrile, phenylpropiononitrile, phenylbutyronitrile, methylphenylacetoneitrile, diphenylacetoneitrile, naphthylacetoneitrile, nitrophenylacetoneitrile, chlorobenzylcyanide, cyclopropanecarbonitrile, cyclohexanecarbonitrile,
35 cycloheptanecarbonitrile, phenylcyclohexanecarbonitrile,

tolylcyclohexanecarbonitrile, and the like.

Preferred are acetonitrile, propionitrile, succinonitrile, butyronitrile, isobutyronitrile, valeronitrile, methyl cyanoacetate, ethyl cyanoacetate, 5 benzonitrile, tolunitrile and chloropropionitrile. More preferred are acetonitrile, propionitrile, butyronitrile and isobutyronitrile, and most preferred is acetonitrile.

As the nitrogen compounds other than nitriles, there may be mentioned, for example, amides such as formamide, N- 10 methylformamide, N, N-dimethylformamide, N,N-dimethylacetoamide, N-methylpyrrolidone, and nitromethane, triethylamine, pyridine, and the like.

As the sulfur compounds, there may be mentioned, for example, dimethyl sulfoxide, sulfolane, and the like.

15 In selecting the organic solvent to be used from among the organic solvents mentioned above, such properties as boiling point and viscosity (e.g. the solvent should have a boiling point which allows appropriate warming for increasing solubility and facilitates a solvent removal 20 from wet masses by drying and solvent recovery from crystallization filtrates and the like (about 30 to 150°C at 1 atm), a melting point such that solidification hardly occurs in handling at room temperature as well as upon cooling to room temperature or below (not lower than about 25 0°C, preferably not lower than about 10°C, more preferably not lower than about 20°C), and a low viscosity (not higher than about 10 cp at 20°C and the like)) are preferably taken into consideration.

The oxidation prevention effect on reduced coenzyme 30 Q₁₀ in a solvent tends to increase in a highly-concentrated solution of reduced coenzyme Q₁₀. Reduced coenzyme Q₁₀ shows high solubility in the above-mentioned organic solvents with high oxidation prevention effect (e.g. hydrocarbons, fatty acid esters and the like). The high 35 solubility makes it possible to handle the highly-

concentrated solution and to promote the oxidation prevention. A preferable concentration of reduced coenzyme Q₁₀ for oxidation prevention at the time of extraction is not particularly limited, but is generally not less than 0.001% by weight, preferably not less than 0.01% by weight, and more preferably not less than 0.1% by weight as the concentration of reduced coenzyme Q₁₀ in the above-mentioned organic solvent. The upper limit is not particularly limited, however, in general, it is not more than 10% by weight.

Among the above-mentioned organic solvents, to extract and recover reduced coenzyme Q₁₀ from wet cells and dry cells of the microbial cells or disrupted product thereof, hydrophilic organic solvents are preferably used. Specifically, there may be mentioned acetone, acetonitrile, methanol, ethanol, 1-propanol, 2-propanol and the like.

Furthermore, among the above-mentioned organic solvents, to extract and recover reduced coenzyme Q₁₀ from the aqueous suspension of the microbial cells or disrupted product thereof, hydrophobic organic solvents are preferably used. Use of such solvents assists the removal of water-soluble substances derived from microorganisms. Many of hydrophobic organic solvents have high oxidation prevention effect as described above, thus are very advantageous.

As the hydrophobic organic solvents, hydrocarbons, fatty acid esters and ethers are preferred.

In the case of the above-mentioned extraction operation, when reduced coenzyme Q₁₀ is extracted from the aqueous suspension of the microbial cells or disrupted product thereof, particularly from the aqueous suspension of the disrupted product, further particularly the case in which the disrupted product is physically treated, by an organic solvent, emulsions tend to be partly formed because of the presence of cell components such as proteins and

phase separation tends to be difficult. Therefore, it becomes important to suppress the formation of emulsions mentioned above and to efficiently carry out extraction.

For that, as an extraction solvent, in addition to
5 the above-mentioned hydrophobic organic solvent, it is preferable to use a hydrophilic organic solvent as an auxiliary solvent in combination.

In this case, the hydrophobic organic solvent is not particularly limited and those mentioned above may be used.
10 Preferred are hydrocarbons, and more preferred are aliphatic hydrocarbons. Among the aliphatic hydrocarbons, those having 5 to 8 carbon atoms are preferably used.

As specific examples of the aliphatic hydrocarbons containing 5 to 8 carbon atoms, there may be mentioned, for
15 example, pentane, 2-methylbutane, hexane, 2-methylpentane, 2,2-dimethylbutane, 2,3-dimethylbutane, heptane, heptane isomers (e.g. 2-methylhexane, 3-methylhexane, 2,3-dimethylpentane, 2,4-dimethylpentane), octane, 2,2,3-trimethylpentane, isooctane, cyclopentane,
20 methylcyclopentane, cyclohexane, methylcyclohexane, ethylcyclohexane, and the like. Particularly preferred are hexane, heptane and methylcyclohexane, and most preferred are hexane and heptane.

The hydrophilic organic solvent to be used in
25 combination with the above-mentioned hydrophobic organic solvent is not particularly limited and those mentioned above may be used. Preferred are alcohols. Among the alcohols, monohydric alcohols having 1 to 5 carbon atoms are preferably used. As specific examples thereof, there
30 may be mentioned, for example, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, isobutyl alcohol, tert-butyl alcohol, 1-pentanol, 2-pentanol, 3-pentanol, 2-methyl-1-butanol, isopentyl alcohol, tert-pentyl alcohol, 3-methyl-2-butanol, neopentyl alcohol, and
35 the like. Particularly preferred are methanol, ethanol, 1-

propanol and 2-propanol, and most preferred is 2-propanol.

The amounts of the above-mentioned hydrophilic organic solvent and hydrophobic organic solvent to be used are not particularly limited. But preferably, as the
5 concentration at the time of extraction, the hydrophilic organic solvent is used in a range of 5 to 50% by volume and the hydrophobic organic solvent is used in a range of 25 to 65% by volume relative to the total volume of the entire solution.

10 In recovering reduced coenzyme Q_{10} , the temperature at the time of extraction is not particularly limited and is generally in a range of 0 to 60°C and preferably 20 to 50°C.

As the extraction method, both batch extraction and
15 continuous extraction (preferably countercurrent multistage extraction) may be used. However, the continuous extraction (preferably countercurrent multistage extraction) is preferable in terms of productivity. The stirring duration in the batch extraction is not
20 particularly limited but is generally not less than 5 minutes. The average retention time in the continuous extraction is not particularly limited but is generally not less than 10 minutes.

In recovering reduced coenzyme Q_{10} , it is preferable
25 to be careful so that reduced coenzyme Q_{10} is not decomposed (e.g. so that reduced coenzyme Q_{10} is not oxidized to oxidized coenzyme Q_{10}). For that, the above-mentioned extraction (including cell disruption) is preferably carried out under an acidic to a weakly basic
30 condition, and more preferably under an acidic to a neutral condition. In the case where a pH is used as an index, although it depends on the contact time, the pH is generally not more than 10, preferably not more than 9, more preferably not more than 8, and still more preferably
35 not more than 7.

By the above-mentioned conditions, an oxidation reaction can be substantially prevented and, optionally, more strictly, the above-mentioned cell disruption and/or extraction are preferably carried out under the condition that reduced coenzyme Q₁₀ is protected from an oxidation reaction. It is preferable to carry out at least the extraction under this condition, and it is more preferable to carry out the disruption and the extraction under this condition.

As "the condition that reduced coenzyme Q₁₀ is protected from an oxidation reaction" means, for example, a deoxygenized atmosphere (an atmosphere of an inert gas such as nitrogen gas, carbon dioxide gas, helium gas, argon gas or hydrogen gas, reduced pressure, a boiling condition); a high salt concentration condition, for example, preferably a condition where salts (e.g. inorganic salts such as sodium chloride and sodium sulfate) are contained in not less than about 5% in an aqueous phase; the condition in the presence of a strong acid (e.g. an acid with a pKa value of not more than 2.5 in an aqueous solution), for example, in the presence of not less than 0.1 mole % of the strong acid relative to 1 mole of reduced coenzyme Q₁₀; and the condition in the presence of an antioxidant, for example, in the concomitant presence of ascorbic acid, citric acid, salts and esters thereof (e.g. not less than 0.1% by weight of them relative to reduced coenzyme Q₁₀). There may also be mentioned a reduction condition (a condition in which oxidized coenzyme Q₁₀ can be converted into reduced coenzyme Q₁₀), for example, a condition involving a contact with a reducing agent such as dithionous acid.

By the above-mentioned culture (fermentation) and extraction, reduced coenzyme Q₁₀ can be suitably produced and recovered. Preferably, an extract containing not less than 70 mole %, preferably not less than 75 mole % of

reduced coenzyme Q₁₀ among the entire coenzymes Q₁₀ is obtained.

Thus-obtained extract containing reduced coenzyme Q₁₀ is optionally purified by column chromatography, reduction treatment, or the like and then subjected to crystallization to obtain high-purity reduced coenzyme Q₁₀ crystals. Incidentally, also in this case, a series of treatment steps are preferably carried out under "the condition that reduced coenzyme Q₁₀ is protected from an oxidation reaction" mentioned above.

In the present invention, oxidized coenzyme Q₁₀ can be produced by oxidizing the above-mentioned microbial cells or disrupted product thereof and then extracting oxidized coenzyme Q₁₀ by an organic solvent, or extracting reduced coenzyme Q₁₀ from the microbial cells or disrupted product thereof by an organic solvent, purifying optionally and oxidizing the resultant to oxidized coenzyme Q₁₀.

The above-mentioned oxidation may be carried out by, for example, mixing reduced coenzyme Q₁₀ (preferably an aqueous suspension of the microbial cells or disrupted product thereof containing reduced coenzyme Q₁₀, an extract containing reduced coenzyme Q₁₀ or the like) with an oxidizing agent (e.g. manganese dioxide or the like) and then, for example, oxidizing the mixture at room temperature (e.g. 30°C) for not less than 30 minutes. In the case where the microbial cells or disrupted product thereof are oxidized, the extraction operation of oxidized coenzyme Q₁₀ can be carried out in the same manner as the above-mentioned extraction operation of reduced coenzyme Q₁₀. Thereby, oxidized coenzyme Q₁₀ can be efficiently recovered. Incidentally, it is not necessary to carry out the recovery of oxidized coenzyme Q₁₀ under "the condition that reduced coenzyme Q₁₀ is protected from an oxidation reaction", which is recommended for the recovery of reduced coenzyme Q₁₀ and the recovery may be carried out in

consideration of general safe operation and the like. The thus-obtained oxidized coenzyme Q₁₀ may be optionally purified by column chromatography or the like, and, finally by conducting crystallization operation, high-purity oxidized coenzyme Q₁₀ crystals may be obtained.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a schematic diagram of a countercurrent 3-step continuous extraction apparatus used in Example 8.

BEST MODE FOR CARRYING OUT THE INVENTION

The following examples illustrate the present invention in further detail. These examples are, however, by no means limitative of the scope of the present invention.

(Example 1)

Various coenzyme Q₁₀-producing microorganisms shown in the following Tables 1 to 3 were cultured with shaking (amplitude: 2 cm, 310 reciprocation/min) at 25°C for 72 hours in 10 mL of culture media [(glucose: 20 g, peptone: 5 g, yeast extract: 3 g, malt extract: 3 g)/L, pH: 6.0] using test tubes (inner diameter: 21 mm, entire length: 200 mm), and the obtained broth were optionally concentrated. Under a nitrogen atmosphere, in the concomitant presence of 3 parts by volume of isopropanol and 18.5 parts by volume of n-hexane relative to 10 parts by volume of the broth, the obtained solutions were vigorously shaken for 3 minutes using 10 parts by volume of glass beads (425 to 600 μm) to carry out cell disruption and extraction. The obtained hexane phases were evaporated (at 40°C) under reduced pressure and analyzed by high performance liquid chromatography (HPLC) to determine the ratio and the production amount of reduced coenzyme Q₁₀.

HPLC conditions

Column: YMC-Pack 4.6 × 250 mm (manufactured by YMC.
Co., Ltd.)

Mobile phase: methanol/n-hexane = 85/15

5 Flow rate: 1 mL/min

Detection: UV 275 nm

The results are shown in Tables 1 to 3. The ratio of
reduced coenzyme Q₁₀ means a mole percentage value of the
10 ratio of reduced coenzyme Q₁₀ relative to the total of
oxidized coenzyme Q₁₀ and reduced coenzyme Q₁₀ on the basis
of the areas of the peaks of reduced coenzyme Q₁₀ and
oxidized coenzyme Q₁₀ and the ratio of the mole absorption
coefficients thereof (1 : 7.5).

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Table 1

Strain name		Upper stand: Ratio of reduced coenzyme Q10 (%) Lower stand: Production amount of reduced coenzyme Q10 (μ g/ml)
5	Agrobacterium tumefaciens IFO 13263	82 7
	Agrobacterium radiobacter ATCC 4718	78 7
10	Aspergillus clavatus JCM 1718	83 2
	Acetobacter xylinum IFO15237	77 2
	Aminobacter aganouensis JCM 7854	70 3
	Agromonas oligotrophica JCM 1494	75 2
15	Acidiphilium multivorum JCM 8867	73 3
	Bulleromyces albus IFO 1192	72 2
	Bullera armeniaca IFO 10112	85 7
	Brevundimonas diminuta JCM 2788	82 5
20	Cryptococcus laurentii IFO 0609	79 6
	Chionosphaera apobasidialis CBS 7430	71 2
	Candida curvata ATCC 10567	74 3
	Cerinosterus luteoalbus JCM 2923	79 5
25	Exisophiala alcalophila JCM12519	77 3
	Exobasidium gracile IFO7788	79 2
	Fellomyces fuzhouensis IFO 10374	70 2
	Filobasidiella neoformans CBS 132	88 2
30	Filobasidium capsuloigenum CBS 1906	82 3
	Geotrichum capitatum JCM 6258	77 3
	Graphiola cylindrica IFO 6426	75 4
	Gluconobacter suboxydans IFO 3257	86 6
35	Kockovaella imperatae JCM 7826	78 2

Table 2

		Upper stand: Ratio of reduced coenzyme Q10 (%) Lower stand: Production amount of reduced coenzyme Q10 (μ g/ml)
5	Kurtzmanomyces nectairei IFO 10118	79 2
	Lalaria cerasi CBS 275.28	75 2
	Leucosporidium scottii IFO 1212	88 6
	Legionella anisa JCM 7573	73 3
	Methylobacterium extorquens JCM 2802	72 2
10	Mycoplana ramosa JCM 7822	80 2
	Oosporidium margaritiferum CBS2531	76 2
	Pseudomonas denitrificans IAM 12023	85 8
	Pseudomonas shuylkilliensis IAM 1092	84 6
	Pseudozyma aphidis CBS 517.23	79 5
15	Paracoccus denitrificans JCM 6892	83 5
	Petromyces alliaceus IFO 7538	72 2
	Rhodotorula glutinis IFO 1125	79 7
	Rhodotorula minuta IFO 0387	74 8
	Rhodosporidium diobovatum ATCC 1830	86 4
20	Rhizomonas suberifaciens IFO 15212	82 2
	Rhodobium orientis JCM 9337	80 2
	Rhodoplanes elegans JCM9224	74 2
	Rhodopseudomonas palustris JCM2524	90 6
	Rhodobacter capsulatus SB 1003	95 6
25	Sporobolomyces holsticus IFO 1034	72 9
	Sporobolomyces pararoseus IFO 0471	93 8
	Sporidiobolus johnsonii IFO 1840	73 7
	Saitoella complicata IFO 10748	97 9

Table 3

	Strain name	Upper stand: Ratio of reduced coenzyme Q10 (%)
		Lower stand: Production amount of reduced coenzyme Q10 (μ g/ml)
5	Schizosaccharomyces pombe IFO 0347	90 8
	Sphingomonas parapaucimobilis IFO 15100	78 7
	Sporotrichum cellulophilum ATCC 20493	73 6
10	Sympodiomyopsis paphiopedili JCM 8318	80 6
	Sterigmatosporidium polymorphum IFO 10121	72 2
	Sphingomonas adhesiva JCM 7370	80 3
15	Tapharina caerulescens CBS 351.35	81 2
	Tremella mesenterica ATCC 24438	89 3
	Trichosporon cutaneum IFO 1198	95 8
20	Tilletiaria anomala CBS 436.72	75 4
	Tilletia caries JCM 1761	80 3
	Tolyposporium bullatum JCM 2006	73 4
25	Tilletiopsis washintonensis CBS 544	76 2
	Ustilago esculenta IFO 9887	78 2
	Udeniomyces megalosporus JCM 5269	87 2
30	Xanthophyllomyces dendrorhous IFO 10129	84 2
	Xanthobacter flavus JCM1204	80 2
	Paecilomyces lilacinus ATCC10114	80 5
35	Acremonium chrysogenum ATCC11550	75 5
	Hyphomonas hirschiana ATCC33886	72 3
	Rhizobium meliloti ATCC9930	85 10

(Example 2)

Rhodotorula glutinis IF01125 was aerobically cultured at 25°C for 48 hours in a culture medium (peptone: 5 g, yeast extract: 3 g, malt extract: 3 g, glucose: 20 g/L, pH: 6.0). The cells after the culture were collected by centrifugation and suspended in a phosphoric acid buffer solution at pH 7 to which N-methyl-N'-nitro-N-nitrosoguanidine have been added so as to have its concentration of 200 µg/mL. After maintaining the solution at 25°C for 1 hour, the cells were washed for 5 times with a 0.9% NaCl solution and further suspended in a 0.9% NaCl solution. The obtained cell suspension was properly diluted and a colony was to be formed on an agar plate of the above-mentioned culture medium. The production amount and the ratio of reduced coenzyme Q₁₀ in the isolated mutant strain were determined in the same manner as Example 1. The strains having higher production amount and the ratio of reduced coenzyme Q₁₀ as compared with those of wild strains was further mutated repeatedly. As the result, by repeating the mutagenesis for 10 times, mutant strains with productivity of not less than 15 µg/mL were obtained. In this case, the ratio of reduced coenzyme Q₁₀ was not less than 80 mole %.

(Example 3)

Saitoella complicata IFO 10748 was aerobically cultured at 25°C for 72 hours in 10 L of a culture medium (peptone: 5 g, yeast extract: 3 g, malt extract: 3 g, glucose: 20 g/L, pH: 6.0). The obtained cells were disrupted for 2 times at 80 MPa of disruption pressure by a pressure homogenizer (manufactured by Lanni Co.) sealed with nitrogen gas to obtain a cell-disrupted solution. The cell-disrupted solution was subjected to extraction with 30 parts by volume of isopropanol and 40 parts by volume of hexane for 3 times to obtain an extract. The extraction

ratio was 99%. The ratio of reduced coenzyme Q₁₀ was 97 mole %.

(Example 4)

5 When mutant strains of Rhodotorula glutinis IFO1125 were aerobically cultured at 25°C in 10 L of a culture medium (peptone: 10 g, yeast extract: 5 g, malt extract: 3 g, glucose: 20 g/L, pH: 6.0), glucose was fed at the rate of 4 g/h after the lapse of 48 hours to 96 hours (fed
10 glucose amount: 190 g). The production amount of reduced coenzyme Q₁₀ per culture medium was not less than 20 µg/mL and the ratio of reduced coenzyme Q₁₀ was not less than 80 mole %.

15 (Example 5)

 The extract obtained in Example 3 was subjected to solvent substitution with a hexane solution, the resultant was adsorbed in a column filled with silica gel and subjected to development and elution by a solution of n-
20 hexane/diethyl ether (9/1) to obtain a fraction containing reduced coenzyme Q₁₀. Furthermore, the fraction was cooled to 2°C with stirring to obtain a white slurry. All the above-mentioned operations were carried out in a nitrogen atmosphere. The obtained slurry was filtered under reduced
25 pressure, the resulting wet crystals were washed with the development solution same as used above (the temperature of the solvent used for washing was 2°C), and the wet crystals were dried under reduced pressure (20 to 40°C, 1 to 30 mmHg) to obtain 81 mg of white dry crystals. The purity of
30 the obtained crystals was 99.9% and the ratio of reduced coenzyme Q₁₀ was 90 mole %.

(Example 6)

 The extract obtained in Example 3 was subjected to
35 solvent substitution with n-hexane, the resultant was added

with 50 mg of manganese dioxide, and the mixture was stirred at 30°C for 30 minutes. Thus-obtained reaction solution was fractionated and purified in the same manner as Example 5 to obtain 74 mg of high-purity oxidized coenzyme Q₁₀.

(Example 7)

Saitoella complicata IFO 10748 was aerobically cultured at 25°C for 72 hours in 500 mL of a culture medium (peptone: 5 g, yeast extract: 3 g, malt extract: 3 g, glucose: 20 g/L, pH: 6.0). The obtained cells were disrupted for 2 times at 80 MPa of disruption pressure by a pressure homogenizer (manufactured by Lanni Co.) sealed with nitrogen gas to obtain a cell-disrupted solution. The ratio of reduced coenzyme Q₁₀ in the cell-disrupted solution was 97% relative to the entire coenzymes Q₁₀ including oxidized coenzyme Q₁₀. 200 mL of the cell-disrupted solution was mixed with isopropanol and n-hexane at the ratios shown in the first extraction section in the following Table 4 so as to adjust the total solvent amount to be 500 mL and the mixtures were stirred at 40°C for 30 minutes to carry out the first extraction. After completion of the extraction, the resultants were kept standing for 10 minutes and the separated upper layers were collected. The volume ratios of the lower layers (residues) relative to the total solution amounts were defined as indexes of separability and shown as the interface positions in Table 4.

Furthermore, in order to carry out the second extraction, the solvent concentrations of the residual layers were measured and isopropanol and hexane were further added so as to keep the solvent ratios in the entire solutions be the ratios shown in the second extraction section in Table 4. The resulting solutions were stirred at 40°C for 30 minutes. Then, the solutions

were kept standing for 10 minutes and the upper layers were collected in the same manner as described above to determine the solvent concentrations of the residual layers. Isopropanol and hexane were added thereto so as to keep the solvent ratios in the entire solutions be the ratios shown in the third extraction section in Table 4, and the solutions were stirred at 25°C for 30 minutes to carry out the third extraction.

The ratios of the amounts of reduced coenzyme Q_{10} contained in the collected upper layers of each of the first, second and third steps relative to the amount of reduced coenzyme Q_{10} contained in the cell-disrupted solution or the extraction residue before the extraction were defined as the extraction ratios of reduced coenzyme Q_{10} in the respective steps. The calculation results are shown in Table 4. The integrated extraction ratios of reduced coenzyme Q_{10} in the second and third extraction steps are also shown. In any steps, the static separability was excellent and the integrated extraction ratio in the case where extraction was repeated for 3 times was as high as not less than 90% to show high recovery ratio. Particularly, in the case where the isopropanol concentration was adjusted to be not less than 30%, the recovery ratio was as high as not less than 99%.

Table 4

		Solvent ratio (vol%)		Interface position	Extraction ratio (%)	
		Isopropanol	Hexane		Respective extraction ratio	Integrated extraction ratio
5	Case1 First	18.8	52.7	0.492	73.6	73.6
	Second	19.0	52.4	0.624	47.6	86.2
	Third	29.7	41.7	0.645	55.5	93.8
10	Case2 First	31.3	40.2	0.499	90.7	90.7
	Second	37.7	33.7	0.549	83.7	98.5
	Third	40.6	30.9	0.565	40.1	99.1
15	Case3 First	31.3	40.2	0.526	89.0	89.0
	Second	34.1	37.3	0.553	85.8	98.3
	Third	36.8	34.6	0.555	46.6	99.1
20	Case4 First	31.3	40.2	0.526	89.0	89.0
	Second	34.1	37.3	0.553	85.8	98.3
	Third	42.4	29.0	0.644	50.0	99.0
	Case5 First	31.3	40.2	0.526	89.0	89.0
	Second	40.1	31.4	0.595	88.1	98.6
	Third	40.7	30.7	0.593	45.3	99.1
	Case6 First	31.3	40.2	0.526	89.0	89.0
	Second	40.1	31.4	0.595	88.1	98.6
	Third	45.8	25.7	0.663	40.7	99.0

(Example 8)

Saitoella complicata IFO 10748 was aerobically cultured at 25°C for 72 hours in 750 L of a culture medium (peptone: 5 g, yeast extract: 3 g, malt extract: 3 g, glucose: 20 g/L, pH: 6.0). The obtained cells were disrupted for 2 times at 140 MPa of disruption pressure by a pressure homogenizer (manufactured by Lanni Co.) sealed with nitrogen gas to obtain a cell-disrupted solution. The cell-disrupted solution was subjected to continuous extraction by a countercurrent 3-step continuous extraction apparatus shown in Fig. 1. The capacity of the stirring tank was 630 L and the capacity of the static separation tank was 200 L. The cell-disrupted solution was supplied

to the first stirring tank and isopropanol and n-hexane were supplied to respective steps. The supply amount of the cell-disrupted solution was 2 L/min and the supply amounts of isopropanol and n-hexane were adjusted to be 1.3 L/min for isopropanol and 3.7 L/min for n-hexane as the total of the supply amounts in respective steps. In this case, the solvent concentration in respective steps was properly adjusted so that the isopropanol concentration of 5 to 50 v/v % and the n-hexane concentration of 25 to 65 v/v % were kept. The extraction temperature was 40°C and the treatment duration was 6 hours. At the point after the lapse of 6 hours, the recovery ratio of reduced coenzyme Q₁₀ extracted from the cell-disrupted solution was calculated on the basis of reduced coenzyme Q₁₀ remaining in the extraction residue in the static separation tank in the third step to find the recovery ratio of 98.9%. The static separation was well carried out during the entire operation period and stable continuous extraction was possible.

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INDUSTRIAL APPLICABILITY

According to the processes of the present invention, reduced coenzyme Q₁₀ can be produced cheaply on the industrial scale by considerably simple steps comprising culturing microorganisms and recovering reduced coenzyme Q₁₀. In addition, oxidized coenzyme Q₁₀ can also be produced by simple processes.

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